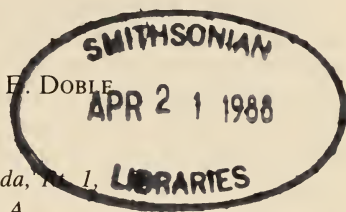


REVIEW

The Variety and Distribution of the FMRFamide-Related Peptides in Molluscs

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INTRODUCTION

Peptide families, like societal kinships, are perceived in terms of morphological, functional, and geneological similarities. Moreover, both sorts of families are poorly delimited, since they comprise a network of relationships extending, from an elementary unit, outward to other existing individuals (or peptides), and backward in time through ancestral lineages. Therefore, although peptide families are often discussed, the underlying concepts remain variable and vague. Since this review is a description of a peptide family, we thought to introduce it with a clarification of our hierarchical terminology.

First, the fundamental unit in peptide associations is the *intragene family*, a set of peptides with similar sequences, all processed from one precursor encoded by a single gene. The sets of enkephalin-containing (EC-) peptides processed from pre-pro-enkephalins A and B are each examples of such fundamental families [1].

Second, within any species, a number of peptides may be clearly related structurally, but be derived from two or more precursors. Such groups are *intraspecific families*, and examples include: the set of EC-peptides generated from 3 precursors in any mammal [1]; the pancreatic polypeptide-related peptides (PP-RPs; including PP, NPY and PYY) in pig [2]; and the ELH-like peptides from two precursors in *Aplysia californica* [3].

Third, homologous peptides from closely related species usually have very similar sequences, but as species and their ancestries become dissimilar, so often do their peptides. Thus, bovine and porcine PP differ by only one residue out of 36, whereas bovine and anglerfish PP have only 16 residues in common [4]. Still, *intrapyletic peptide families* are readily recognized, even when the number of residues common to all members is relatively small. A case in point is the family of 8 peptides related to pigment concentrating hormone of prawns and adipokinetic hormone of insects which has only three invariant common residues (pGlu¹, Phe⁴, and Trp⁸) [5]. *Interphyletic*, or even *universal families* have been proposed [6], but are very difficult to define.

Finally, the functions of peptides are not a consideration in these definitions for, although members of intragene families may have a common function, the functions attributed to related peptides become increasingly heterogeneous at higher hierarchical levels.

In this review, we consider an emerging intrapyletic family of molluscan peptides related to FMRFamide (Phe-Met-Arg-Phe-NH₂). We summarize the major and minor components of this family and their phylogenetic distribution; we describe a methodological key to the characterization of the member peptides, and present some new, exemplary, data about the family in the snail *Helix aspersa*. In the end, we speculate about the ancestry of this peptide family and about its extension to other phyla.

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MAJOR FMRFamide-RELATED PEPTIDES (FaRPs)

FMRFamide

FMRFamide had its origin in several observations that ganglion extracts from various molluscs would increase the force of contraction of isolated hearts of venerid clams, and that this excitation was not inhibited by the serotonin antagonists methysergide and 2-bromo-d-lysergic acid diethylamide [7–9]. Frontali *et al.* [10] began to purify this cardioactivity by gel chromatography with Sephadex G-15. Four cardioexcitatory peaks were separated from the well-studied molluscan neurotransmitters, and one of them—peak C, which was retained on the column and eluted just after the salt volume—occurred in every species tested in all of the major groups of molluscs; and it was a potent cardioexcitor and antiarrhythmic agent [8, 9]. The source of this activity was sought, with persistence and vigor, in the ganglia of *Macrocallista nimbosa*, the sunray venus clam, and was finally identified as the tetrapeptide, FMRFamide [11]. FMRFamide has since proven to be ubiquitous in Mollusca and an invariably major component of FMRFamide-like immunoreactivity in that phylum (Table 1).

The pulmonate heptapeptides

Studies on the FMRFamide-like activity in the ganglia of *Helix aspersa* started soon after the isolation of FMRFamide, and the first was an attempt to localize FMRFamide to identifiable neurones using bioassay [12]. This investigation led to the preliminary conclusion that FMRFamide itself is not present in *Helix*, but is replaced by a novel FMRFamide analog with a modified N-terminal and considerably more potency on the *Helix* heart than the parent peptide [13]. Using HPLC for fractionation and radioimmunoassay for detection, three major peaks of FMRFamide immunoreactivity were detected in *Helix* ganglia [14]. Attention was focused on the most retained (i.e., non-polar) one, which was identified, finally, as a heptapeptide with an N-terminal pyroglutamyl residue [14]. The amino acid sequence of this peptide is: pGlu-Asp-Pro-Phe-Leu-Arg-Phe-NH₂

(pQDPFLRFamide), the methionyl residue of FMRFamide being replaced by leucine, and it is, indeed, much more potent than FMRFamide on the *Helix* heart [15]. The remaining two major peaks in *Helix* are FMRFamide and a partially characterized analog of pQDPFLRFamide, observations that will be discussed below in some detail.

Several additional species of pulmonate snails and slugs have since been examined. In each case, the extracts contain three major FaRPs in roughly equal quantities. One of these is always FMRFamide, as mentioned above; the others are all heptapeptides analogous to pQDPFLRFamide (Table 1). The common heptapeptide sequence is X-DPFLRF-NH₂, and peptides with pGlu, Gly, Ser and, probably, Asn as the N-terminal residue have been identified thus far (Table 1).

Although only a small number of species has been sampled, a clear difference has emerged between the heptapeptides of the two major orders of Pulmonata—the Basommatophora (mostly limnic snails) and the Stylommatophora (mostly terrestrial snails and slugs). First, in the stylommatophoran pulmonates—represented in Table 1 by *Helix*, three other snails from disparate families, and the slug *Limax maximus*—one of the pair of heptapeptides is always pQDPFLRFamide; the other is indeterminate (Table 1). Moreover, the chromatographic peaks of stylommatophoran heptapeptides are easily distinguished (see section on Methodology, below).

Among the basommatophorans, two species have been studied in some detail: the false limpet *Siphonaria pectinata*, a primitive member of the order [16], and the more advanced snail *Lymnaea stagnalis* [17]. These two species representing the upper and lower ends of the phylogenetic range, as well as other lymnaean snails that have been tested, all contain the Gly¹ analog GDPFLRFamide as one of their two heptapeptides (Table 1). Thus, GDPFLRFamide appears to be characteristic of the Basommatophora. As to the second heptapeptide in this pulmonate order, the Ser¹ and the Asn¹, have been identified; but pQDPFLRFamide has never been detected in a limnic snail (Table 1). The basommatophoran heptapeptides are also characteristically difficult to resolve, and thus to characterize (see section on Methodology,

TABLE 1. Major and minor FMRFamide-related peptides (FaRPs) in molluscs

CLASS SUBCLASS Order Species	Major FaRPs			Minor FaRPs		Refs.
	FMRFamide	Heptapeptides ^a (X-DPFLRFamide)		FLRFamide	Others	
		1°	2°			
POLYPLACOPHORA						
<i>Acanthopleura granulata</i>	+	—ni—		ni		d
BIVALVIA						
PTERIOMORPHIA						
<i>Geukensia demissa</i>	+	—ni—		+		[19]
HETERODONTA						
<i>Macrocallista nimbosa</i>	+			ni		[11]
GASTROPCDA						
PROSOBRANCHIA						
Mesogastropoda						
<i>Pomacea paludosa</i>	+	—ni—		+	SGFLRF	[19]
Neogastropoda						
<i>Busycon contrarium</i>	+	—ni—		+		[19]
OPISTHOBRANCHIA						
<i>Aplysia brasiliana</i>	+	—ni—		ni		[20]
<i>Aplysia californica</i>	+	—ni—		+*	nt-GYLRFa*	[21]
PULMONATA						
Basommatophora						
<i>Siphonaria pectinata</i>	+	Gly	Asn	ni		[16]
<i>Lymnaea stagnalis</i>	+	Gly	Ser	ni		[17]
<i>Stagnicola palustris</i>	+	—c—		ni		[19]
<i>Helisoma sp.</i>	+	Gly	ni	ni		d
Stylommatophora						
<i>Strophocheilus oblongus</i>	+	pGlu	c	ni		d
<i>Succinea campestris</i>	+	pGlu	c	ni		[19]
<i>Limax maximus</i>	+	pGlu	c	ni		[18]
<i>Helix aspersa</i>	+	pGlu	Asn ^b	ni		[15], d
<i>Cepaea nemoralis</i>	+	pGlu	c	ni		[19]
CEPHALOPODA						
<i>Octopus vulgaris</i>	+	—ni—		+	YGGFMRFamide	[26, 43]
<i>Octopus bimaculoides</i>	+	—ni—		+	ni	[46]

+ Unambiguous identification.

ni Sought but not identified.

* Predicted from gene sequence.

^a Where heptapeptides were identified, the N-terminal residue (X-) is listed. The primary heptapeptide (1°) is characteristic of the order; the 2° heptapeptide is not.^b Prediction based on amino acid composition and homology.^c Unresolved peak eluting on HPLC (ACN/TFA system) with Ser-, Asn, and Gly-DPFLRFamide.^d Previously unreported.

below).

MINOR FaRPs

A number of minor immunoreactive peaks always occur in chromatograms of ganglion extracts. Some appear to be FMRFamide analogs secreted in small amounts, some may arise as artifacts of purification, and some may be processing intermediates.

FLRFamide

This peptide was discovered in the mesogastropod, *Pomacea paludosa*, the apple snail. *Pomacea* contains very large quantities of FMRFamide-like immunoreactivity in its ganglia: several hundred picomoles per snail. Most of this immunoreactivity is due to FMRFamide, but the Leu² analog FLRFamide is also present at 10–20% the level of the parent peptide [19]. FLRFamide has since been identified in several other molluscs, including bivalves, cephalopods, and always at the same low levels (Table 1). This tetrapeptide is difficult to identify in most animals, not only because it is present in small amounts, but also because it is less reactive in the radioimmunoassay (see Methodology section below).

Although FMRFamide was easily characterized in the opisthobranch *Aplysia braziliana*, FLRFamide was not detected [20]. Yet the FMRFamide precursor in *Aplysia californica* does appear to generate one copy of FLRFamide [21]. FLRFamide has also never been detected in a pulmonate, but nothing is yet known about the gene or precursor in these organisms. These gaps in our information will be discussed toward the end of this review.

SGFLRF

This is the tentative sequence for another, very small peak of immunoreactivity from *Pomacea*. Notwithstanding the low immunoreactivity, the amino acid levels were high enough to determine a composition — Ser, Pro, Phe₂, Leu, Arg — so this peak is probably not amidated [19].

YGGFMRFamide

Met-enkephalin-Arg⁶-Phe⁷ (YGGFMRF) was

first discovered in the adrenal chromaffin granules and in the striatum of the ox [22], and has been reported to occur in molluscs [23]. Although YGGFMRF has no effect as an agonist on the standard bioassay for FMRFamide, its C-terminally amidated analog is approximately equipotent with FMRFamide [24, 25].

A peak of FMRFamide-like immunoreactivity eluting with YGGFMRFamide was detected in ganglion extracts from *Octopus vulgaris* [26]. Its identification as YGGFMRFamide was based on its immunoreactivity with various antisera, its HPLC retention times in various solvents, and its activity in an opiate binding assay (Table 1).

Artifacts of purification

FMRFamide is susceptible to oxidation, but the oxidized peptide has only slight biological activity and may not be detected by bioassay. However, oxidized FMRFamide is still immunoreactive, and can be separated from the unoxidized peptide on HPLC, if gradient elution is used. Deliberate oxidation of *Helix* ganglion extracts with hydrogen peroxide showed that the FMRFamide peak behaved as expected for authentic FMRFamide; i.e., the peak at the normal elution time for FMRFamide disappeared, and the peak at the position of oxidized FMRFamide increased [15]. This shift of the peak with oxidation, or the appearance of both peaks as a doublet, are good, practical indicators of the occurrence of FMRFamide.

In an analysis of ganglion extracts from the pulmonate *Lymnaea stagnalis*, Ebberink *et al.* [17] detected the unamidated forms of the two major heptapeptides in a single batch of material. A conservative interpretation of this observation is that the heptapeptides were enzymatically deamidated during the purification. This notion is supported by the diminished height of the immunoreactive heptapeptide peak in the same preparation.

Another peak that might be expected to arise during the purification of pulmonate extracts is one containing Pro-Phe-Leu-Arg-Phe-NH₂. This peptide could arise from the cleavage of the extremely acid-labile Asp-Pro bond of the heptapeptides.

THE INTRAPHYLETIC FAMILY

The phylogenetic distribution of major and minor FaRPs within the Phylum Mollusca is summarized diagrammatically in Figure 1. The heterogeneity of the intraphyletic family, due primarily to the taxonomic restriction of the pulmonate heptapeptides, is evident. Also clear, however, is that the picture in Figure 1 is incomplete in two ways.

First, some minor classes — Aplacophora, Polyplacophora, and Scaphopoda — have been ignored or inadequately studied, primarily because of practical considerations such as supply or size. More critical is the absence of information about peptides in taxa closer to the divergence of the three gastropod subclasses. For example, a study of the families Onchidiidae and Veronicellidae, which are considered systellommatophoran pul-

monates by some authors [28] and gymnophilan opisthobranchs by others [29] should be particularly rewarding.

Second, although the characterizations of SGFLRF in Mesogastropoda, and of YGGFMRFamide in Cephalopoda, are not complete, their occurrence does suggest that a variety of minor FaRPs are widely distributed in molluscs and await discovery. Some new information about minor FaRPs in *Helix* is set out below (and Table 1).

METHODOLOGY

The identification of a particular peptide depends, not only upon the species and tissue examined, but also upon the methods of extraction and purification followed and the bioassay used to monitor these procedures. Therefore, we review

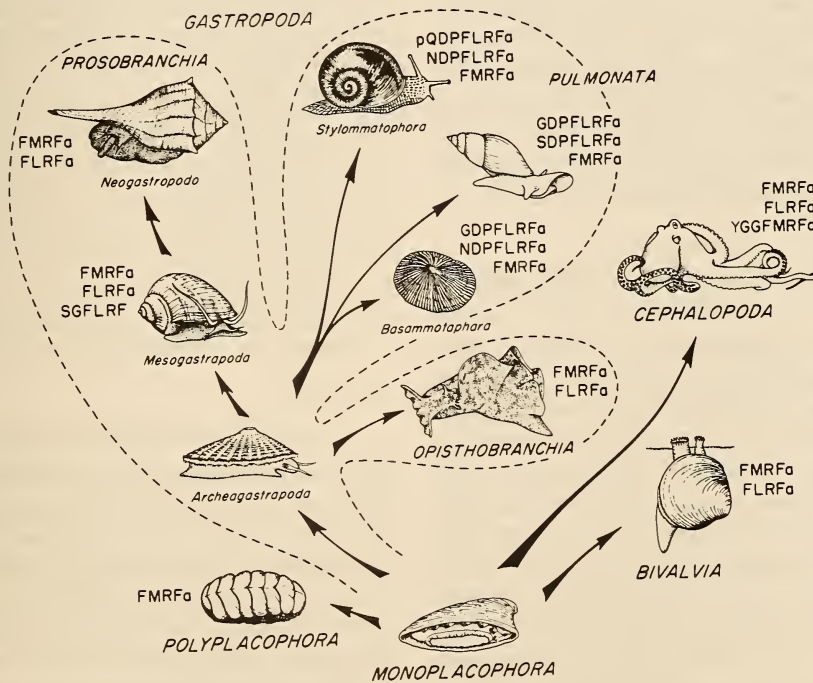


FIG. 1. The distribution of FMRFamide-related peptides in the Mollusca, showing, especially, the ubiquity of FMRFamide and the delimited occurrence of the pulmonate heptapeptides (general sequence, X-DPFLRFamide). The phylum is assumed to radiate from primitive molluscs similar to tryblidian monoplacophorans. An archeogastropod is pictured to provide continuity, but no member of this subclass has been examined for its peptides. a, amide.

in brief the specific steps involved in characterizing the FMRFamide-related peptides of molluscs.

Assay systems: bioassay

The classical clam heart assay [30] was used throughout the identification of FMRFamide, but the isolated radula protractor of *Busycon contrarium*, prepared as first described by Hill [31], was also introduced and increasingly relied upon. The radula protractor muscle is not only very sensitive to FMRFamide, but is also much more selective than the clam heart [32]; e.g., this muscle is contracted by FMRFamide and its analogs, but by few other substances, and especially not by 5-hydroxytryptamine (5-HT), a ubiquitous major component of molluscan ganglion extracts. Moreover, the radula protractor muscles from small *Busycon* can be isolated in a microbath and used to assay very small volumes of material [33].

Other molluscan hearts, muscles, muscular organs, and nerve cells respond to FMRFamide, and the structure-activity relations (SAR) of the peptide have been studied on some of them [34–36]. But most of these preparations are neither sufficiently sensitive nor convenient to serve as bioassays. In any event, radioimmunoassay (RIA) has become the system of choice for monitoring the purification of FMRFamide-like peptides.

Assay systems: radioimmunoassay

Several RIAs for FMRFamide, using different antisera, were developed soon after the tetrapeptide was sequenced [e.g., 37–41], but only one of them has been used to identify all of the new molluscan FMRFamide-like peptides [41]. This antiserum was raised to a conjugate of thyroglobulin with YGGFMRFamide. The rationale for doing this was that, since the peptide could only be conjugated through its N-terminal, the glycyl residues would serve as a spacer, extending the C-terminal FMRFamide sequence away from the protein carrier and allowing more specificity for the peptide hapten. The expected result was realized: the antiserum, in contrast to ones made to conjugates of FMRFamide itself, recognizes (though not equally) all of the residues in the FMRFamide sequence.

The cross-reactivity of the antiserum has been

studied in detail and compared with those of other antisera [41, 42]. Three of its features have especially contributed to the analysis of chromatographic data (illustrations in Table 2): 1) The antiserum is unusually specific for the N-terminal phenylalanyl residue; even the substitution of a tyrosine in this position drastically lowers the cross-reactivity. 2) Analogs extended at the N-terminal are more reactive in the RIA than is FMRFamide itself, especially if the extension has a glycyl residue analog. Other antisera, and most bioassays, react with extended peptides about as strongly as with FMRFamide, or slightly worse [41]. 3) Substitutions for the methionyl residue tend to be relatively benign; in particular, oxidation of the methionine or its replacement with a leucyl residue has relatively little effect on immunoreactivity.

TABLE 2. Immunoreactivity of selected synthetic peptides in an RIA for FMRFamide

Peptide sequence	Cross-reactivity*
FMRFamide	1.00
FLRFamide	.25
YMRFamide	.007
FMRYamide	.002
YGGFMRFamide	50.0
YGGFLRFamide	10.0
pQDPFLRFamide	2.0
SDPFLRFamide	.6
GDPFLRFamide	.6

* Relative to FMRFamide taken as 1.00.

Extraction

In most peptide investigations, an early purification step can be achieved through the judicious choice of a species from which very large ganglia (or other tissues) can be dissected cleanly and conveniently. In contrast, phylogenetic studies (e.g., on the evolution of a peptide family) place special demands on extraction methods; the procedures must apply to any relevant species, no matter how small, heavily shelled, or otherwise inconveniently constructed.

Acetone has proven to be a very salutary extraction medium. If the ganglia can be dissected out, the FaRPs will be extracted with a sufficient

degree of purity that gel filtration can be eschewed, and high performance liquid chromatography (HPLC) performed directly (details below). More important, significant quantities of immunoreactive FMRFamide can be recovered simply by steeping whole animals, with their shells, in acetone at -4°C . Recently, we were able to characterize the three major FaRPs in the primitive pulmonate snail *Siphonaria pectinata* by extracting thousands of these abundant, but very small organisms [16].

Notwithstanding the success of acetone marination, other methods such as acid extraction have also been used to isolate FMRFamide-like peptides with good results [43].

Gel chromatography

Although this methodology would seem to have been rendered obsolete by HPLC, fractionation on Sephadex G-15 remains a useful component in the purification of FaRPs (Fig. 2A). The major advantage is that all of the known peptides in this family elute together, with good recovery, in the classic peak C of Frontali *et al.* [10], thereby effecting an early and impressive purification. Still, in instances when a very clean dissection has been possible, gel filtration is omitted. In such cases, the extracts are injected directly onto an HPLC column (e.g., Waters C18 Radial Pak) and eluted with an ammonium acetate/*n*-butanol buffer system. If the extract is clean enough, this chromatographic step can separate out several major and minor peaks (method and example in Fig. 3A).

HPLC: a chromatographic key

The identification of the components of peak C by HPLC may require two steps, with the procedure and results dependent on the taxon of the sample species. The possibilities and their outcomes are summarized in Figure 2, a kind of methodological key.

The first step is reverse-phase HPLC with elution in a gradient system (ACN/TFA) comprising 0.072% trifluoroacetic acid (TFA, the aqueous solvent) and 80% acetonitrile with the same concentration of TFA (ACN, the organic solvent; details of the ACN/TFA system in Fig. 2 and

[16]). When extracts of non-pulmonate molluscs are chromatographed in this system, FMRFamide and oxidized FMRFamide appear as a pair of immunoreactive peaks, and FLRFamide occurs in a very small peak (Fig. 2Bi), sometimes only a shoulder. A similarly unambiguous result obtains when a stylommatophoran extract is chromatographed in the ACN/TFA system: the immunoreactive peaks of pQDPFLRFamide and the second heptapeptide are well separated and both are equal in height to the FMRFamide peak (Fig. 2Biii).

Basommatophoran extracts in the ACN/TFA system, however, yield a single immunoreactive peak in addition to, and about twice as large as, that of FMRFamide plus oxidized FMRFamide (Fig. 2Bii). This result is caused by the virtual coelution of GDPFLRFamide (i.e., the basommatophoran characteristic) and all other heptapeptides except pQDPFLRFamide which is never found in basommatophorans. The two heptapeptides can be resolved by HPLC with elution in another gradient system (ACN/ PO_4) comprising 5 mM sodium phosphate, pH 7.0, and acetonitrile (details in Fig. 2 and [16]). The ACN/ PO_4 system clearly separates GDPFLRFamide from its Asn¹ analog in *Siphonaria* (Fig. 2Ci), or from its Ser¹ analog in *Lymnaea* (Fig. 2Cii).

THE FMRFAMIDE-RELATED PEPTIDES OF *HELIX*

The ganglion extracts of *Helix aspersa* were the first to be examined after FMRFamide was discovered, and it was in this snail that the first pulmonate heptapeptide to be sequenced—pQDPFLRFamide—was identified. Nevertheless, the second heptapeptide in *Helix* has not yet been characterized, and most of the minor immunoreactive peaks have never been analyzed. Recently, however, we purified 400 circumoesophageal ganglionic rings, dissected out of snails in St. Andrews, Scotland. The results of this study, reported here, have given us further insight into the FMRFamide-like peptides of *Helix* and the evolution of the peptide family.

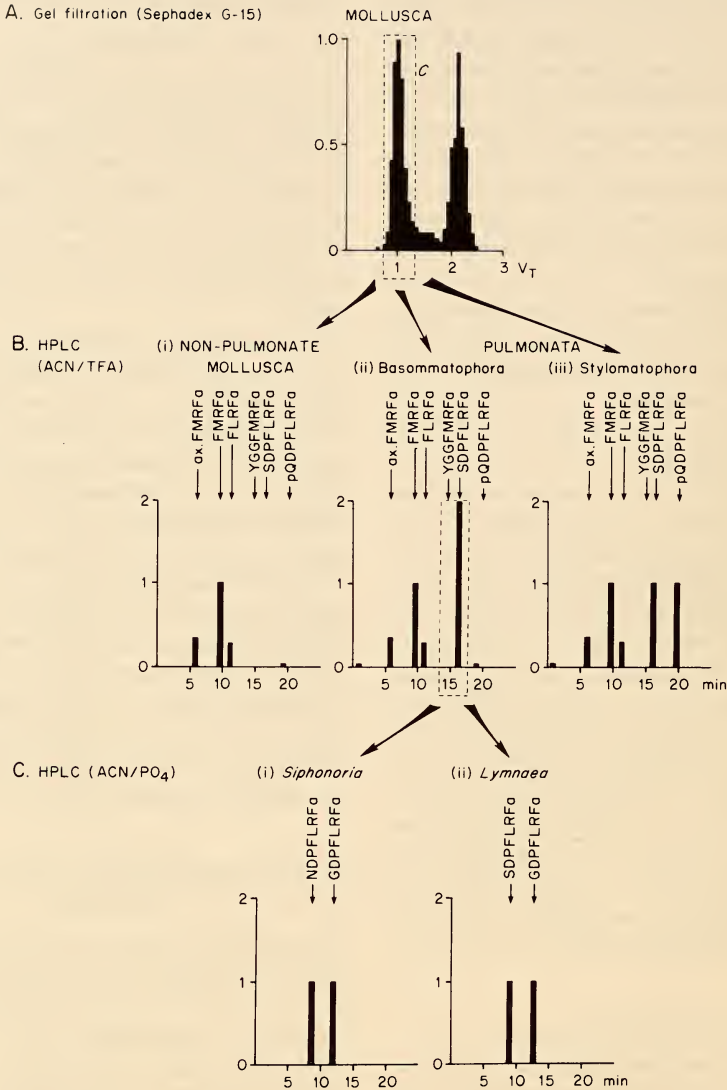


FIG. 2. A diagrammatic scheme for purifying and characterizing FMRFamide-related peptides (FaRPs) from molluscan ganglion extracts, including a set of idealized chromatographic profiles characteristic of particular taxa. The profiles are plots of FMRFamide-like immunoreactivity with time. A: The extract is applied to G-15 and eluted with 0.1 M acetic acid. B: Immunoreactive peak C, comprising fractions that elute at one column volume (V_T), is applied to reverse-phase HPLC, and eluted with a solvent system including acetonitrile (ACN) and trifluoroacetic acid (TFA). The nonpulmonate FaRPs (Bi), and those of stylomatophoran pulmonates (Biii), are resolved in the ACN/TFA system; basommatophoran heptapeptides are not (Bii). C: The complex immunoreactive peak of basommatophoran heptapeptides can be resolved by HPLC in a solvent system containing acetonitrile and phosphate buffer (Ci and Cii). The buffer systems are described in the text and the legend for Fig. 3, as well as [16].

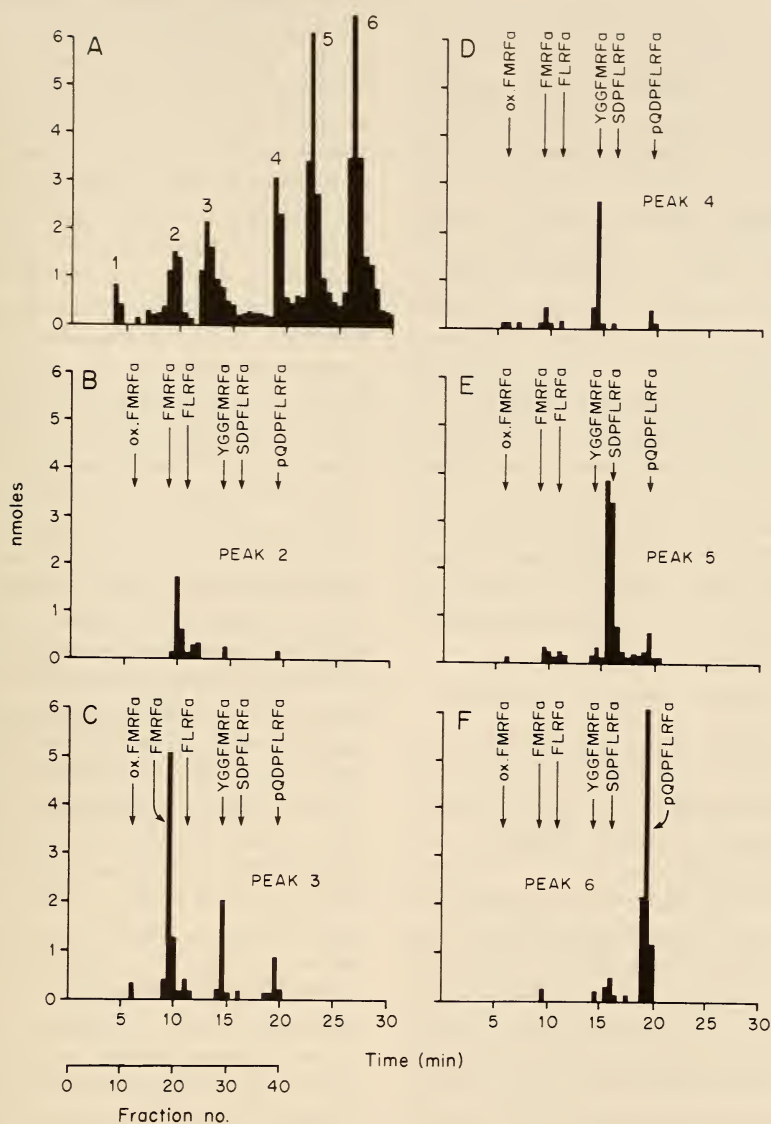


FIG. 3. Purification and chromatographic identification of an acetone extract of snail (*Helix aspersa*) ganglia. A: HPLC fractionation on a Waters C18 Radial-Pak column; elution with a solvent system consisting of 0.5M ammonium acetate, in 0.1M acetic acid (pH 5.5), and *n*-butanol. The butanol was first held constant at 4% (10 min), and was then increased to 8% over 20 min. The solvent was pumped at 4 ml/min; 0.5 min fractions were collected. The FMRFamide-like immunoreactivity in each fraction (plotted on the ordinate) was determined from radioimmunoassay (RIA) of an aliquot. The six major peaks of immunoreactivity are labeled (1-6), and these designations are used in B-D: The six peaks were separately pooled and lyophilized and re-chromatographed. HPLC was on a Waters C18 Microbondapak column; elution with a gradient (12-40% over 30 min) of acetonitrile in 6mM trifluoroacetic acid. Solvent flow, 2 ml/min; fractions, 0.5 min. Immunoreactivity was measured by RIA, and UV absorbance was monitored at 210nm. The positions of several standards, determined in separate but similar runs, are indicated.

Extraction

Each ganglionic ring was dropped into acetone as soon as it was removed from the animal in Scotland, and the mixture was kept frozen until it was brought to St. Augustine, Florida, for further processing. The well-travelled acetone was decanted, centrifuged, and the supernatants saved. The ganglia were then homogenized (Polytron) in aqueous acetone (80%), the mixture centrifuged, and the acetone removed from the combined supernatants on a rotary evaporator. The small volume of aqueous solution left in the flask was forced through a C18 cartridge (Waters Sep-Pak) which was washed with water and the active material then eluted with methanol. The methanol was also removed by rotary evaporation, and the residue was taken up in water for injection directly onto the HPLC column (ammonium acetate/butanol system). This, then, is an instance of a clean dissection obviating a preliminary fractionation on Sephadex G-15.

Purification

Six, clear, immunoreactive peaks (labeled 1-6 in Fig. 3A) were the major features in the elution pattern of this first HPLC step. *Peak 1* eluted at the expected position of oxidized FMRFamide and was not further characterized.

Peaks 2 and 3 both eluted near the position of FMRFamide itself. Either peak could have contained FMRFamide, but they were well-resolved, so we kept them separate and re-chromatographed each with the ACN/TFA buffer system (Fig. 3A, B). This new separation showed that the bulk of the material eluting with FMRFamide is in *Peak 3* (Fig. 3C), but there also appears to be some in *Peak 2* (Fig. 3B).

The fractions constituting the two peaks were hydrolyzed and the amino acid composition of each fraction analyzed. The fractions that appeared to contain FMRFamide in *peak 3* had, in fact, the composition expected of the tetrapeptide (e.g., 3-19 in Table 2). In fact, this composition completes the identification of FMRFamide in *Helix aspersa*; previous characterizations had been based only on the elution times of the oxidized and unoxidized peptides [15, 44].

The results of re-chromatographing *peak 2* were not so clear (Fig. 3B). The largest peak, at about 10 min, included the small amount of FMRFamide occurring in *peak 2*, but contained very low levels of amino acids. In contrast, the minor peak of immunoreactivity, comprising fractions 23 and 24, which eluted near the position of FLRFamide, contained high levels of a small number of amino acids. The composition, Asx₂, Pro₁, Tyr₁, Leu₁, Arg₁, Phe₁ (Table 2; 2-24), however, is not consistent with the minor peak being FLRFamide. Moreover, the immunoreactivity observed was low: only 2% of that to have been expected were FLRFamide present at the level suggested by the amino acid analysis. Finally, since FLRFamide elutes after FMRFamide in the ammonium acetate/butanol system, it should have appeared (had it been present) in *peak 3*, rather than in *peak 2*.

When *peak 4* from the butanol system was re-chromatographed with the ACN/TFA buffer system, a large peak of immunoreactivity resulted (Fig. 3D), but its component fractions contained no corresponding peak of amino acids. Thus, this peak could be an RIA artifact [44], but it might also represent a substance with considerably more immunoreactivity than FMRFamide.

Peak 5, upon re-fractionation, yielded a clear peak of immunoreactivity (Fig. 3E) with a coincident peak of UV absorbance (not shown) and high amino acid levels (Table 3, 5-32). The analysis is not unambiguous, but the most reasonable composition — Asx₂, Pro₁, Phe₂, Leu₁, Arg₁ — is identical to that determined for an analogous heptapeptide peak in *Siphonaria pectinata* [16]. Moreover, it is also similar to the composition of the minor peak in *peak 2*, except that, in the latter, a tyrosinyl residue replaces one of the phenylalanines (compare 5-32 with 2-24 in Table 3).

Finally, when the most retained peak in the ammonium acetate/butanol system — *peak 6* — was re-chromatographed in the ACN/TFA system, the elution time corresponded well to the previously characterized heptapeptide, pQDPFLRFamide (Fig. 3F).

Summary of Helix FaRPs

Price [14] had previously shown that *Helix* ganglia contain three major peaks of FMRFamide-

TABLE 3. Amino acid analysis of peak immunoreactive fractions from *Helix aspersa*

Amino acid	Fraction 3-19 ^a		Fraction 2-24 ^a		Fraction 5-32 ^a	
	nmol	ratio	nmol	ratio	nmol	ratio
Phenylalanine	1.543	1.83(2)	3.132	1.18(1)	3.505	1.70(2)
Arginine	.873	1.03(1)	2.654	1.00(1)	2.082	1.01(1)
Aspartic Acid	.141	.17(0)	5.124	1.93(2)	2.910	1.41(1-2)
Proline	.117	.14(0)	2.622	.99(1)	2.057	1.00(1)
Leucine	.205	.24(0)	2.832	1.07(1)	2.450	1.19(1)
Methionine	.845	1.00(1)	.192	.07(0)	.435	.21(0)
Tyrosine	.072	.09(0)	1.940	.73(1)	.192	.09(0)
Glycine	.129	.15(0)	.977	.39(0)	.567	.28(0)
Serine	.150	.18(0)	.757	.29(0)	1.144	.56(0)

^a An extract of ganglia was fractionated on HPLC (ammonium acetate/butanol system). The 6 peaks were then rechromatographed (TFA/ACN system) (see Fig. 3). Fractions 19, 24 and 32, from rechromatographed peaks 3, 2 and 5, respectively, were hydrolyzed, and the amino acids analyzed.

like immunoreactivity. One of them is FMRFamide and another is the classic heptapeptide pQDPFLRFamide [15]. The remaining major peak (peak 5, Fig. 3E) still eludes full characterization. But we hypothesize that it is the heptapeptide Asx-DPFLRFamide, a sequence also proposed for its analog in *Siphonaria pectinata* which was discussed earlier in this review.

The two minor peaks in *Helix* ganglia differ from the major peaks in that their levels of immunoreactivity are not equivalent to the levels of peptide determined by amino acid analysis. One of these minor peaks (fractions 23 and 24 in re-chromatographed peak 2; Fig. 3) is much less immunoreactive than FMRFamide although the peptide levels are similar. In contrast, peak 4 contains little peptide, and thus seems to be more immunoreactive than FMRFamide. The characteristics of the antiserum used in the RIA (Table 2) suggest that a *decrease* in immunoreactivity would reflect some change in the tetrapeptide core of FMRFamide. An *increase* in immunoreactivity would be brought about by changes in the molecule (e.g., N-terminal extension) that would make it more like YGGFMRFamide, the antigen to which the antiserum was raised. If the minor peak of peak 2 had the sequence: Asx-DPYLRF-NH₂, suggested by its composition (Table 3; 2-24), it would (in spite of the N-terminal elongation) surely be less immunoreactive due to the substitu-

tions of the leucyl and, especially, the tyrosinyl residues (see Table 2). Peak 4 is considered further, below.

FMRFamide GENES AND PRECURSORS

The Aplysia precursor

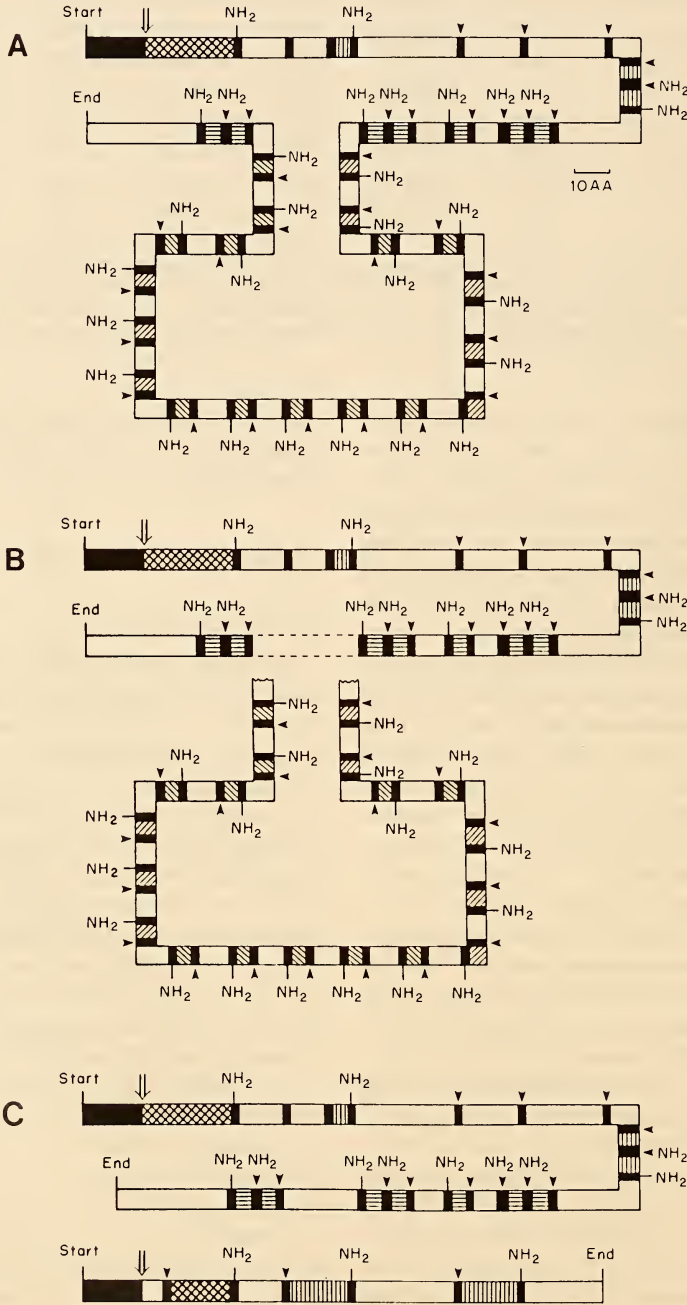
The inhomogeneity evident in the intraphyletic family of FMRFamide-related peptides should be explicable in terms of the precursors of the constituent intragene families. To date, however, only one FMRFamide precursor, that from *Aplysia californica*, has been studied. The gene encoding this precursor has been cloned and its sequence determined [21]. The precursor (Fig. 4A) is about 600 amino acids long and contains: 28 putative copies of the FMRFamide sequence, one copy of FLRFamide, and one copy of another peptide ending in -Gly-Tyr-Leu-Arg-Phe-NH₂ (designated nt-GYLRFa to indicate that it is at the n-terminal of the precursor and is amidated).

The number of functional copies of FMRFamide arising from this precursor is still in question because many of the sequences would have to be processed out by cleavage at a single lysine residue, a mechanism unknown for other peptide precursors [45]. However, the report of Lehman *et al.* [20], that FMRFamide was the only identifiable FaRP in *Aplysia* ganglion extracts, is consistent

with FLRFamide and nt-GYLRFa being very minor components of the intraspecific family.

The basic molluscan precursor

One striking feature of the *Aplysia* gene (and the precursor it encodes) is its high content of



repetitive sequences (Fig. 4a; [21]). Long stretches of the precursor are composed of repeated 15 or 16 amino acid segments, each containing one FMRFamide sequence. Many of the repeats are identical at the amino acid level, and several are even completely identical at the nucleotide level. These repeats must represent relatively recent iterative events, and we can therefore approximate the ancestral gene by simply deleting the segments containing most of the similar repeats (Fig. 4B). This gene encodes a truncated precursor containing FLRFamide and nt-GYLRFa, but fewer than ten copies of FMRFamide.

Such a precursor would account reasonably well for the ratio of FMRFamide to FLRFamide seen in both a prosobranch, *Pomacea paludosa* [19], and in two species of octopods, *Octopus bimaculoides* [46] and *Octopus vulgaris* [43]. This correlation therefore supports our proposition that the back-extrapolation in Figure 4B resembles the basic molluscan precursor, which is conserved in modern bivalves, cephalopods and most gastropods, and is ancestral to the opisthobranch and pulmonate precursors.

The number of FMRFamide-related peptides actually processed from the putative basic molluscan precursor—e.g., three distinct peptides as in *Aplysia*, or only the two (FMRFamide and FLRFamide) detected to date—remains unknown. However, Price [19] reported the occurrence, in *Pomacea*, of a minor peptide which appeared to be SGFLRF (Table 1); and K. H. Voigt has found other FLRFamide-related pep-

tides in *Octopus* (personal communication). We conclude, therefore, that the proposed basic precursor probably contains at least as many distinct peptides as that of *Aplysia*.

The pulmonate precursor

If we assume that our analysis of the minor peaks of *Helix* is both correct and applicable to all pulmonate species, and if we add those data to our information about the major immunoreactive peaks of pulmonates (Table 3), we find that the family of FMRFamide-related peptides in the Subclass Pulmonata falls into two branches, as follows. The *heptapeptide branch* contains three FLRFamide analogs, all present at about equal levels: XDPFLRFamide, X*DPFLRFamide, and X*DPYLRamide (e.g., in *Helix*, X is pGlu, and X* is Asn). As described above, the first two are much more immunoreactive than the third which is the minor peak of fraction 2 of *Helix* (Fig. 3B). What we will call the *basic branch* of the family contains FMRFamide and a highly immunoreactive peptide present at very low levels (*Helix* peak 4, Fig. 3D). Of these two branches, it is the heptapeptide one that is novel and that distinguishes the pulmonate FaRP family from those of the other molluscs.

The precursor proteins in the pulmonates, and the genes that encode them, are completely unknown at present. Nevertheless, a reasonable conjecture about the molecular basis for the pulmonate family is possible. We propose that the pulmonates contain two distinct genes and precur-

FIG. 4. Hypothetical representation of the FMRFamide precursors in molluscs and their derivation by back-extrapolation from the known *Aplysia* precursor. A: The FMRFamide precursor of *Aplysia* redrawn from [21]. The initiation methionine at the N-terminal is labeled "Start," and the following hydrophobic signal sequence is solid black. The large arrow shows where the signal sequence is cleaved from the precursor, and the nt-GYLRFa peptide follows immediately (cross-hatched). The bold black bars are basic amino acid residues, potential processing points. Simple cleavage points are at the small arrows; cleavage sites with amidation signals (glycyl residue) are indicated by NH₂. The single copy of FLRFamide is vertically hatched. Copies of FMRFamide in the unique (basic) region of the precursor are horizontally hatched; those in the iterative regions are diagonally hatched. The bends in the diagram have no physical meaning. B: Hypothetical basic molluscan FMRFamide precursor. The most iterative region of the *Aplysia* precursor has been deleted, leaving FLRFamide, and about 10 copies of FMRFamide. (All symbols are as in A.) C: Two hypothetical precursors accounting for the FMRFamide-related peptides of *Helix* and other pulmonates. One precursor is presumed to be similar to the basic molluscan precursor (upper diagram). It yields FMRFamide, FLRFamide and an analog of nt-GYLRFa (possibly peak 4 of *Helix*). The second precursor, unique to the pulmonates, produces three heptapeptides, two immunoreactive (in *Helix*, pQDPFLRFamide and putative NDPFLRFamide) and one poorly immunoreactive (putative NDPYLRamide from *Helix* peak 2). (All symbols as in A.)

sors giving rise to the two branches of the peptide family (Fig. 4C). (The alternative notion, that a single precursor gives rise to all of the pulmonate peptides, ignores both the minor peaks of immunoreactivity and observations that the relative sizes of the various peaks vary markedly from tissue to tissue [44]).

First, a precursor resembling the conserved basic molluscan precursor will produce multiple copies, about ten, of FMRFamide. We suggest that it also produces a peptide with the partial sequence -GFLRFamide. This peptide—a close analog of nt-GYLRFa in the *Aplysia* precursor—would have a greatly enhanced immunoreactivity and, therefore, though its concentration would be about one tenth that of FMRFamide, it would appear to be more abundant. This peptide would account for fraction 4 of *Helix* (see Fig. 3A, D). Finally, the basic precursor should also give rise to a single copy of FLRFamide, a peptide not yet detected due to its scarcity and low immunoreactivity. Clear demonstrations of FLRFamide at a level one tenth that of FMRFamide, and of the proposed -GFLRFamide, would go far toward confirming this portion of the hypothesis.

The origin of a distinct heptapeptide gene is not at all clear. Two bits of evidence—that the heptapeptides are all FLRFamide analogs, and that the phenylalanyl residue is replaced with a tyrosine in both the pulmonate heptapeptide of low immunoreactivity (X*DPYLRFamide) and the nt-GYLRFa peptide of *Aplysia*—suggest that the heptapeptide gene could have arisen by duplication from the N-terminal portion of the basic molluscan gene. Alternatively, the heptapeptide gene, rather than being unique to the pulmonates, may occur in all molluscs. Its products might not have been detected for two reasons: they might be expressed at low levels, or they might be processed in a non-immunoreactive form (i.e., unamidated). An example of the latter possibility might be the putative peptide SGFLRF in *Pomacea* [19].

THE INTERPHYLETIC FAMILY

FMRFamide, *sensu stricto*, has been unambiguously identified in a variety of molluscan species (Table 1), but never convincingly in any non-

mollusc. Indeed, until very recently, the few non-molluscan FMRFamide-like peptides sequenced have only had the final two residues of the C-terminal [47, 48]—or at most the final three [49, 50]—in common with either FMRFamide or FLRFamide.

Within the last several months, however, three very FMRFamide-like peptides have been sequenced in non-molluscs, and they are all N-terminally extended analogs of FLRFamide. One of these peptides is pGlu-Asp-Val-Asp-His-Val-FLRF-NH₂ (leucomyosuppressin), isolated from extracts of cockroach heads [51]. Two other peptides, characterized from the pericardial glands of the American lobster, are: Ser-Asp-Arg-Asn-FLRF-NH₂ and Thr-Asn-Arg-Asn-FLRF-NH₂ [52]. That these closest non-molluscan analogs of FLRFamide occur in the Arthropoda, a protostomous phylum with close affinities to the molluscs, is strongly suggestive of an interphylum peptide family.

The novel arthropodan peptides most resemble the two molluscan peptides that are processed out of the N-terminus of the *Aplysia* precursor; i.e., nt-GYLRFa and FLRFamide. Moreover, the N-terminus is especially lacking in obvious redundancies and would thus appear to be the oldest part of the precursor. We therefore speculate that the primeval "FMRFamide" precursor present in the common ancestor of the molluscs and arthropods had only FLRFamide-related sequences.

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